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DATE: July 12, 2002

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		Total Pages (Incl. This Cover Page):	**18**

RE: Application No. 09/830,986

MESSAGE:

Examiner Willis:

Thank you for allowing us to discuss this matter with you on Tuesday, at 10:00 AM.

Enclosed is an article which we would like to discuss during the interview. The article is by the instant inventors and was submitted after the priority date of the instant application to Pharmaceutical Research. We have also enclosed the "General Comments", which were received based upon the original submission. Please note the reference to the idea as being "brilliant."

We would also like to discuss the Hunt et al article, which was discussed in our last response.

Please call me if you have any questions, or any topics which you would like us to address during our interview.

We look forward to discussing this matter with you next week. Thank you for your time.

Sincerely
Donna Meuth

Moss 1,977,642 Oct. 23, 1934

Page 11, "Vinylite, Series V resins for Surface Coatings", pub. 1939 by Carbide & Carbon Chem. Corp., N. Y. City

Widmer et al. 2,197,357 Apr. 16, 1940

Moore 2,218,474 Oct. 15, 1940

[1] This appeal is but one of four cases collectively presented here for determination. Because the parties are identical, the cases very closely related, and the same questions of patentability involved, the four cases were argued at the same time. As described in the Solicitor's brief--

> All of the applications relate to coating compositions adapted for application to metal objects and to be baked thereon to form a hard transparent film. All of the compositions contain a melamine-formaldehyde resin which has been reacted with an alcohol and in addition they contain one or another of the following: cellulose acetate, ethyl cellulose or polyvinal acetate acetal. The claims recite certain minimum proportions or a range of proportions of the last named materials to the resin a certain minimum proportion of the formaldehyde to melamime in the resin, and certain limitations as to the alcohol employed. In general the rejection of the claims has been on the ground that recited proportions were ascertained by mere routine experimentations without the exercise of invention.

Of the cited references in the instant case, the patent to Moss, No. 1,815,444 discloses an adhesive liquid coating composition containing a mixture of cellulose acetate and a synthetic resin compatible therewith in a volatile solvent, in which the proportions of cellulose acetate and synthetic resin, as described in example 1, range from 1:9 to 9:1.

In Moss, No. 1,977,642, a coating composition is disclosed containing cellulose acetate to which natural or synthetic resins may be added to improve not only the strength but also the adhesive qualities of the composition. Moss recites an extensive list of suitable synthetic resins.

The publication of the Carbide & Carbon Chemical Corporation, "Vinylite," discloses compatibility ranges of "Vinylite resin," and numerous other film-forming materials established by compatibility tests.

Widmer et al. discloses coating compositions made of melamine-formaldehyde-alkyl resins which may be used as lacquers. Many species of the composition are described in 49 examples printed in the specification.

Example 1 shows a mixture of formaldehyde and melamine in the molal ratio of 8 to 1 and the compound thus obtained is added to butyl alcohol, as described in example 9, which in turn, "may be added, for example, to nitrocellulose lacquers in order to lend them hardness and filling capacity."

The patent to Moore relates to coating compositions such as varnishes, lacquers, "and in general, to film-forming compositions for the production of films requiring good strength, hardness, and adhesive power." Blends of melamine-formaldehyde-resin and alkyd resin may be mixed "with other materials of the same or different classes such as cellulose esters or ethers including nitrocellulose, cellulose acetate."

Moore is illustrated by twelve examples which describe certain of the more specific features of his invention. Example 1, for instance, describes a melamine-formaldehyde resin, reacted with n-butanol. This resin is mixed with nitrocellulose to make a lacquer. The molal ratio of formaldehyde to melamine described in example 1 is 4.5:1.

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Donna Meuth

11

Mucoadhesive polymers in drug delivery systems

Graham Hunt, Patrick Kearney and Ian W. Kellaway, The Welsh School of Pharmacy, University of Wales Institute of Science and Technology, P.O. Box 13, Cardiff CF1 3XF, UK

1. INTRODUCTION

Bioadhesive polymers have been employed in both surgery and dentistry for many years. Such polymers include the well-documented 'super glues', the esters of α-cyanoacrylates [1,2], which have found applications ranging from repair of osteochondral fractures to capping extraction wounds in dentistry. Other synthetic bone glue candidates [3,4] have included polyurethanes, epoxy resins, acrylates and polystyrene. Often the mechanism of bonding involves the formation of covalent bonds with the target tissue to provide a permanent or semipermanent linkage.

In the development of oral controlled-release dosage forms, considerable benefits may ensue from the use of bioadhesive polymers providing relatively short-term adhesion between the drug delivery system and the mucus or epithelial cell surface of the gastrointestinal (GI) tract. Bonding will therefore involve secondary forces such as hydrogen bonds or London–van der Waals forces. Mucoadhesives may therefore be regarded as a specific class of bioadhesives. Polymer candidates would need to be non-toxic and non-absorbable, adhere rapidly to wet tissues and release the incorporated drug in a controlled manner. Further refinements would seek to achieve specificity as to the site of adherence within the GI tract.

One of the principal objectives of oral controlled drug delivery is to achieve once-a-day dosing, which reduces patient non-compliance and generally improves drug therapy. The vagaries of the GI transit profile therefore present a challenge to the design of such delivery systems. Although transit times of 8–10 h from mouth to colon may be regarded as normal in humans, nevertheless considerable variations are known to exist. Most of this variation occurs in the gastric emptying of dosage forms, which is influenced by both form type and diet [5]. Small intestinal transport appears less dependent on such factors [6]. It is for this reason that control of the gastric emptying of dosage forms by mucoadhesive formulations is an attractive proposition.

The ability to localize a drug delivery system in a selected region of the tract would, in general, lead to improved bioavailability; more especially for drugs exhibiting narrow windows of absorption or instability in certain sections of the tract. Intimate contact with the target absorption membrane should lead to optimization of both the extent and rate of drug absorption. Alternative mechanisms for the control of GI transit of the dosage form, for example through manipulation of particle size and density [7], together with the use of fibrous materials, have not, in the main, been successful.

This paper will attempt to review the development of mucoadhesive polymers, from a consideration of the target tissue, polymer characteristics, *in vitro* testing techniques and the limited amount of *in vivo* evaluation reported to date.

2. CHARACTERISTICS OF THE TARGET TISSUE

There are two ways a material may adhere to a mucosal surface, either by binding to the tissue itself or by association with the mucus coat which is intimately associated with the tissue surface. The characteristics of these surfaces are quite different and the distinction ought to be emphasized.

2.1 The mucus layer

The stomach mucosa is the primary target in the development of a mucoadhesive-based sustained/retarded-release action as gastric retention will be the main mechanism in delaying the rapid absorption which occurs once a formulation reaches the specialized absorptive areas of the small intestine. Throughout the GI tract the mucosal surface is comprised of columnar epithelial cells, the morphology of which changes as the tract is descended. In the stomach there are specific mucus-secreting glands in the cardiac and pyloric regions [8], which serve to coat incoming food boli and hence reduce the possible abrasive action. Mucus cells are also found in the necks and depths of the acid-secreting gastric pits where they form a protective buffer zone around the stream of acid. The mucus coating over the rest of the stomach surface is maintained by the surface columnar epithelial cells and secretion is stimulated by mechanical and chemical irritation [9]. The mucus layer also serves to protect the gastric epithelium from the action of secreted acid and proteolytic enzymes [10]. The layer is usually continuous but can be disrupted under the action of certain irritant substances and an ineffective mucus layer is usually associated with conditions of gastric ulceration [11].

In the small intestine the Brunner's glands of the duodenum supply a copious mucus secretion to protect against the high acid content of the chyme released from the stomach. Mucus throughout the rest of the GI tract is provided by the goblet cells, which represent an increasing fraction of the total epithelial surface towards the colon. They constitute 10% by volume of the mucosa in the upper small intestine, 40% in the lower small intestine and 55% in the colon [12]. Mucus is stored in granules in the apical supra-nuclear half of these cells, which causes them to distend into the characteristic 'goblet' shape. As with the surface cells of the gastric mucosa,

must wet the surface (classical adhesion theory), hence these factors are interrelated. Table 1 lists, in order of decreasing mucoadhesive ability, the major polymers studied by Smart *et al.* [28] and is similar to the results of Chen and Cyr [35] and Park and Robinson [36].

If these polymers are considered in the light of the criteria listed above, it is possible to identify certain factors that would appear to be main contributors to the mucoadhesive properties of the materials. Fig. 1 shows the structures of the polymers in Table 1 and a comparison of their chemical and structural features allows establishment of the inherent broad contributory factors.

In accordance with the theory that secondary bond formation is the principal source of mucoadhesion, those polymers with carboxyl groups present are all, without exception, mucoadhesive. The carboxyl group in its unionized form is capable of strong H-bond formation, and in its ionized form also able to interact electrostatically. However, the functional groups on the polymer backbone should not be in such close proximity that they interfere with each other (e.g. by intramolecular H-bonding). As the carboxyl concentration along a polymer chain decreases, for example in moving from sodium alginate to Karya gum to gelatin, the mucoadhesive strength also decreases.

The effect of other secondary bond-forming groups (e.g. hydroxyl, ether oxygen, amine) on the mucoadhesive properties of the polymers above is not as definite as for the carboxyl group. The cellulosic polymers have an abundance of hydroxyl and ether groups along their length, yet the mucoadhesion exhibited bears little relationship to this, since the cellulose derivatives are found throughout Table 1. Further variation in the possible rank order of the celluloses is introduced by varying the degree of substitution of the polymer.

Another important feature of mucoadhesive molecules is believed to be the ability to form physical bonds principally by entanglement with the substrate molecules. This would appear to be demonstrated by polyethylene oxide (PEO), a linear flexible molecule with minimal secondary bond-forming capacity. Yet at high molecular weights this molecule exhibits a mucoadhesive strength comparable to *McCaud* sodium alginate whose secondary bond-formation ability is far greater. The reason for this could be that the segmental mobility of PEO is extremely high, the ether linkages make for a very flexible backbone, and hence interpenetration into substrate networks is deep and relatively rapid. The effective depth is, however, limited by molecular chain length (i.e. molecular weight) since a short-chain molecule can form fewer entanglements and penetrate to a lesser degree than a larger molecule. This can be illustrated by reference to PEO (molecular weight 40000) and PEG (molecular weight 6000) in Table 1. Primarily the intimacy of interaction between the adhesive and mucus or mucosal tissue is a surface tension phenomenon; hence the lower the contact angle between the adhesive and the mucus/mucosa the better the chances of interaction of the two molecular systems.

The ideal mucoadhesive would arise from a combination of various carefully balanced properties. It must be a polymer of high molecular weight to maximize adhesion through entanglements and van der Waals forces. The segmental mobility of the polymer chain should be high to facilitate rapid and deep penetration into the substrate. The repeating unit of the polymer should contain carboxyl groups and

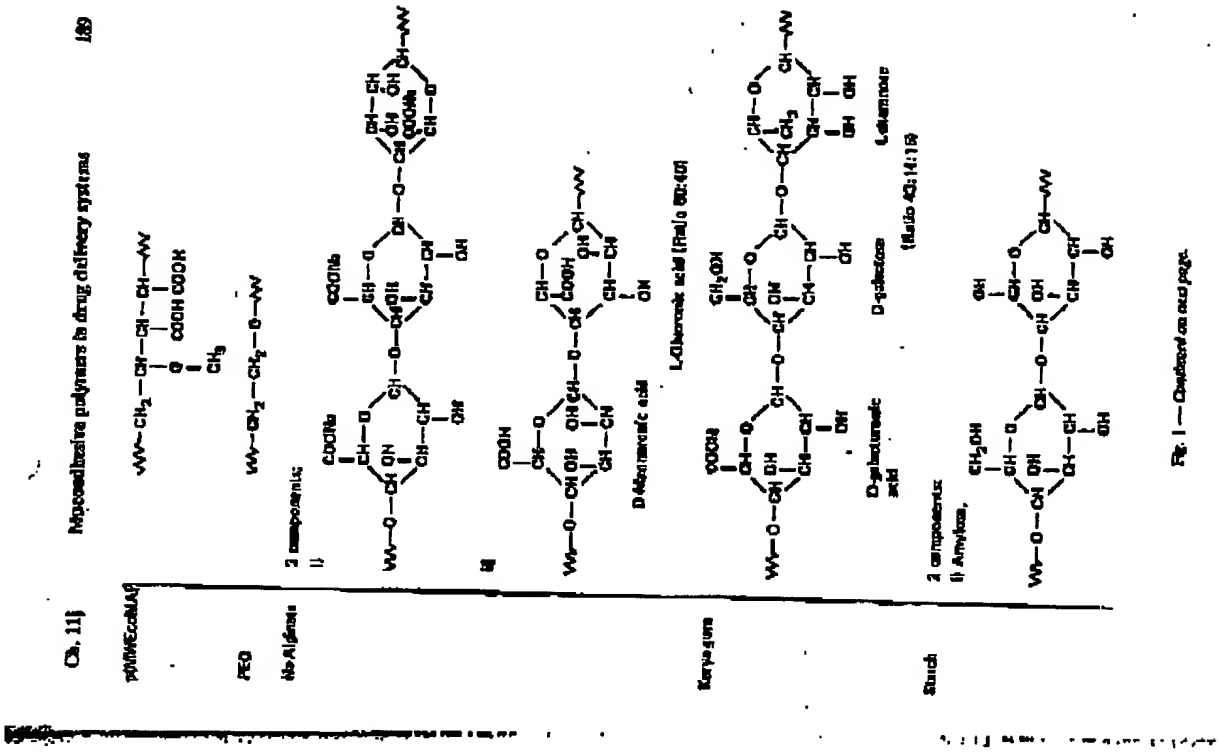
Table 1 — Rank order of mucoadhesive force for various polymers [37]

Test polymer	Mean % adhesive force	Standard deviation
Sodium carboxymethyl cellulose	192.4	12.0
Poly(acrylic acid)	185.0	10.1
Tragacanth	154.4	7.5
Poly(methyl vinylether co-maleic anhydride)	147.7	9.7
Poly(ethylene oxide)	128.6	4.0
Methylcellulose	128.0	2.4
Sodium alginate	126.2	12.0
Hydroxypropylmethylcellulose	125.2	16.7
Karya gum	125.2	4.8
Methylethylcellulose	117.4	4.2
Soluble starch	117.2	3.1
Gelatin	115.8	5.6
Pectin	100.0	2.4
Poly(vinyl pyrrolidone)	97.6	3.9
Poly(ethylene glycol)	96.0	7.6
Poly(vinyl alcohol)	94.8	4.4
Poly(hydroxyethylmethacrylate)	88.4	2.3
Hydroxypropylcellulose	87.1	13.9

other secondary bond-forming groups, principally primary hydroxyl groups and short-chain ethers. This would ensure the potential for adhesion via as many modes as possible.

4. *IN VITRO* TEST METHODS

A number of methods have been employed in an attempt to measure the bioadhesion exhibited by polymers, with some techniques designed specifically for the measurement of mucoadhesion. The methods used to determine the mucoadhesive ability of polymers have measured tensile strength, the closely related peel strength, or a chemical interaction. Chen and Cyr [35] used lap-shear and bending tensile tests to study mucoadhesive formulation. In this method a carriage of weights was attached via a pulley to a strip of plastic bearing the mucoadhesive polymer. The polymer had previously been pressed onto a sample of wet dialysing cellophane, and the time taken for a load of 250g to separate the two materials was measured. A similar procedure using a Chatillon strain gauge was also employed. Peel tests were also carried out, again using a Chatillon strain gauge, on oral mucosa and teeth, and showed similar results to those obtained *in vitro*. The duration of adhesion was also



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pMMAcoMAA

PEO

Na-Alginate

2 components:
I)
II)

Karaya gum

L-Glucuronic acid (Ratio 60:40)

D-Galacturonic acid

D-galactose

Ghatti

2 components:
I) Arabinose,

Fig. 1 — Continued on next page.

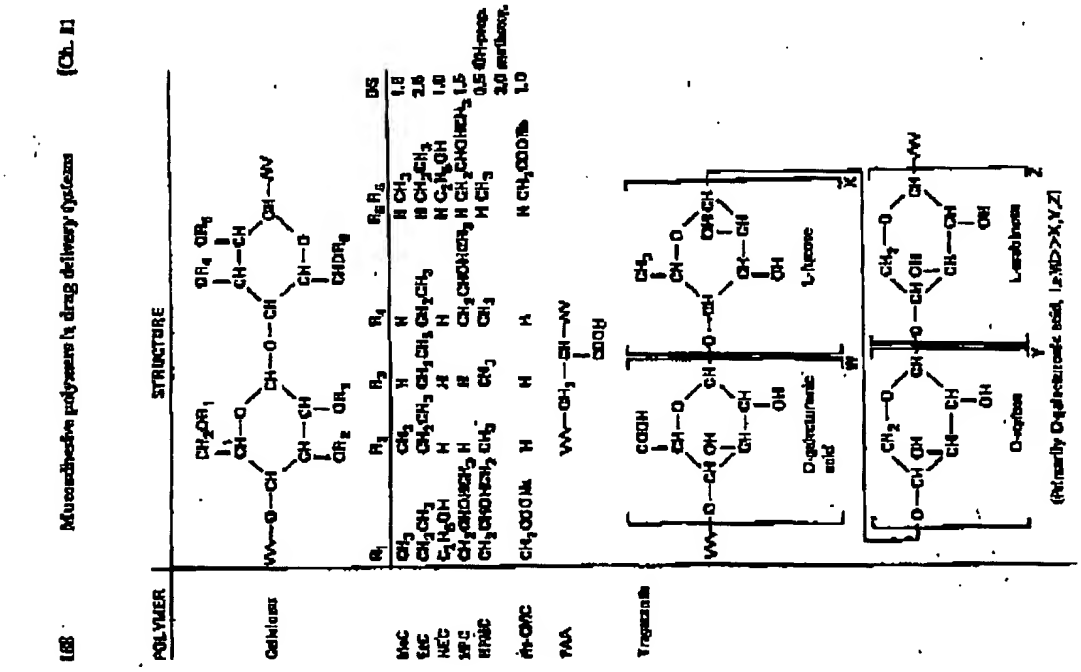

Fig. 1 — Continued on next page.

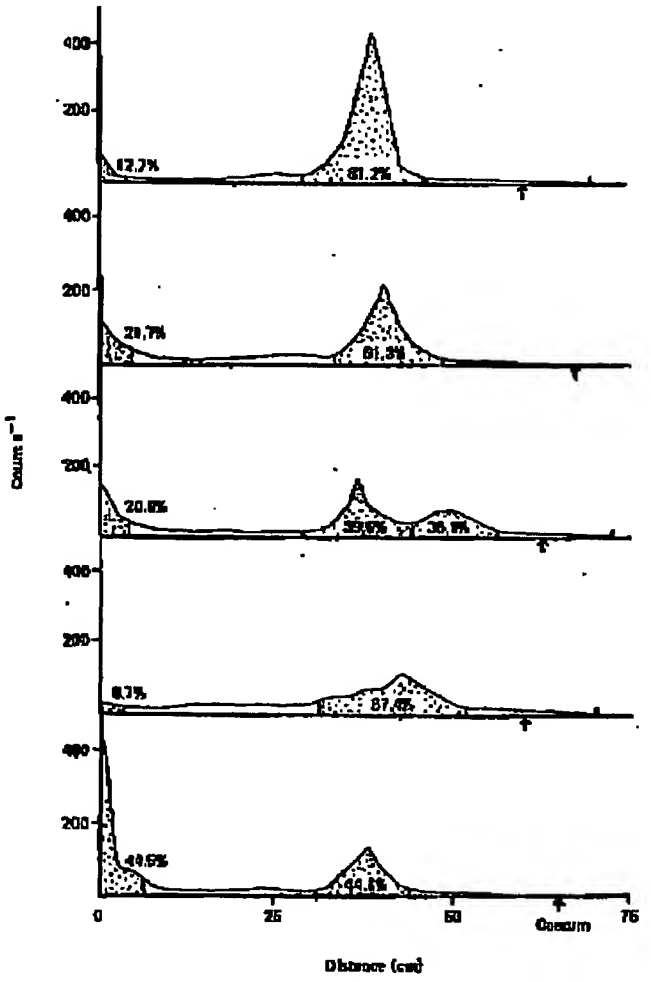

Fig. 3 — The 1-h transit profiles for 9 μm resin particles. The percentage total activity associated with the peaks is for five mice.

associated with particles removed from the stomach, which appears as a peak (occasionally two) between the stomach and the caecum.

The presence of P75 SCMC-adsorbed film did not significantly affect GI transit, whilst a Carbopol 934P film almost completely arrested gastric emptying of the test particles (Table 2). Such effects may be due to mucosa-adhesion or to gastric

Table 2 — The effect of adsorbed films on gastrointestinal transit of 9 μm particles, 1 h after administration

Adsorbed film	No. of mice in group	% retained in stomach
None	15	29.7±18.4
P75 SCMC	4	38.7±48.4
Carbopol 934	5	97.7±5.2

obstruction caused by particle aggregates. With a sterically stabilised particle, aggregation would not be expected and, indeed, no evidence was observed *in vitro* at gastric pH.

Further evidence for the gastric retention of polyacrylic acid polymers was obtained by Ch'ng *et al.* [41] using ^{51}Cr-Polycarbophil surgically introduced into the stomachs of fasted rats. ^{51}Cr-Polycarbophil was shown to have a significantly slower GI transit compared with ^{57}Cr-poly(methacrylic acid–divinylbenzene) (Table 3).

Table 3 — Rat stomach half-transit times and emptying rate constants of test materials [41]

Test materials	Stomach half-transit time	Stomach emptying rate constant (h^{-1})
^{51}Cr-Normal saline	12 min	3.47
Amberlite 200 resin beads	2 h 15 min	0.31
^{51}Cr-Poly(methacrylic acid–divinylbenzene)	6 h	0.12
^{51}Cr-Polycarbophil	12 h 15 min	0.06

These authors also claim to have achieved bioadhesion within the small intestine. A bioadhesive formulation of chlorothiazide resulted in plasma levels in the rat which were of longer duration, and greater bioavailability compared with a sustained-release bead formulation and the drug presented as a powder [42].

The gastric emptying of Polycarbophil has also been examined in dogs [33], when only 8% of the dose was shown to empty within 90 min. Further canine studies [44] showed that only 30% of a 90 g Polycarbophil meal emptied in 4 h. The presence of such a large mass of polymer may have elicited motor activity of the stomach with subsequent reduced gastric emptying.

An alternative to using animal models is to employ the oral mucosa of volunteers [37]. Mucoadhesive polymer candidates can be converted to granules by standard processing techniques. Granules (20–25) were placed onto the oral mucosa below the lower lip and counted at regular intervals until dislodged, or for a maximum time of 45 min. Between counts the mouth was closed, the tongue kept behind the teeth and mouth movements, other than swallowing, were kept to a minimum. Photographs were taken on each occasion to permit ease of counting, and to provide an estimate of the rate of swelling of the polymer granules. Table 4 illustrates some results obtained

Table 4 — The duration of mucosa-adhesion and swelling of test granules

Granules	Time for 50% loss (min)	Time for 100% loss (min)	% swelling after 5 min	Max % swelling	Time of max % swelling (min)
Ethylcellulose	2	3	—	—	—
P75 SCMC 50%/ hard paraffin 50%	10	15	33	61	10
P75 SCMC 20%/ pHEMA 80%	7	20	58	98	10
P75 SCMC 50%/ ethylcellulose 50%	6	16	136	159	6
Carbopol 934	—	>45	213	—	—
Carbopol 934 50%/stearic acid 50%	—	>45	35	94	15

for various test formulations. Ethylcellulose, a non-mucoadhesive polymer, was rapidly cleared from the mucosa. Other attempts to prolong the action of P75 SCMC by reducing the water uptake (addition of paraffin and ethyl cellulose) were only partially successful. This was also true for P75 SCMC blends with the hydrogel pHEMA. The clearance rates were of good reproducibility, although considerable variation was found for swelling measurements. In this particular test, Carbopol 934P performed very much better than P75 SCMC.

In a volunteer study employing three subjects, Khosla and S. S. Davis (personal

communication) demonstrated that Polycarbophil administered with labelled pellets did not reduce the gastric emptying rate of the pellets compared with pellets without the Polycarbophil.

ACKNOWLEDGEMENTS

G. H. gratefully acknowledges SERC and Merrell Pharmaceuticals Ltd, Egham, Surrey for financial support.

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London, UK

Deutsche Bibliothek Cataloguing-in-Publication Data

Drug delivery systems : fundamentals and techniques
/ ed. by P. Johnson and J. G. Lloyd-Jones. –
Weinheim ; New York : VCH ; Chichester : Horwood,
(Ellis Horwood series in biomedicine)
ISBN 3-527-26565-9

NE: Johnson, P. [Hrsg.]

ISSN 0930-5367

British Library Cataloguing in Publication Data

Drug delivery systems : fundamentals and techniques. —
(Ellis Horwood series in biomedicine, ISSN 0930-5367).
1. Delayed-action preparations
I. Johnson, P. (Peter) II. Lloyd-Jones, J.G.
615'.6 RS201.C64

Library of Congress CIP data available

Published jointly in 1987 by
Ellis Horwood Ltd., Chichester, England
and VCH Verlagsgesellschaft mbH, Weinheim, Federal Republic of Germany

Distribution:

Great Britain and Ireland: VCH Publishers (UK) Ltd., 8 Wellington Court, Wellington Street, Cambridge CB1 1HW (Great Britain)

USA and Canada: VCH Publishers, Suite 909, 220 East 23rd Street, New York, NY 10010-4606 (USA)

Switzerland: VCH Verlags-AG, P.O. Box, CH-4020 Basel (Switzerland)

All other countries: VCH Verlagsgesellschaft, P.O. Box 1260/1280, D-6940 Weinheim (Federal Republic of Germany)

ISBN 3-527-26565-9 (VCH Verlagsgesellschaft) ISBN 0-89573-580-6 (VCH Publishers)

Printed in Great Britain by Unwin Bros. of Woking

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Pharmaceutical Research, Vol. 16, No. 6, 1999 *Research Paper*

Polymers with Thiol Groups: A New Generation of Mucoadhesive Polymers?

Andreas Bernkop-Schnürch,[1,2] Veronika Schwarz,[1] and Sonja Steininger[1]

Received December 24, 1998; accepted March 5, 1999

Purpose. To improve the mucoadhesive properties of polycarbophil by the introduction of sulfhydryl groups.
Methods. Mediated by a carbodiimide, cysteine was covalently bound to polycarbophil (PCP) forming amide bonds between the primary amino group of the amino acid and the carboxylic acid moieties of the polymer. The amount of covalently attached cysteine and the formation of disulfide bonds within the modified polymer were determined by quantifying the share of thiol groups on the polymer conjugates with Ellman's reagent. The adhesive properties of polycarbophil-cysteine conjugates were evaluated *in vitro* on excised porcine intestinal mucosa by determining the total work of adhesion (TWA).
Results. Depending on the weight-ratio of polycarbophil to cysteine at the coupling reaction, e.g., 16:1 and 2:1, 0.6 ± 0.7 μmole and 5.3 ± 2.4 μmole cysteine, respectively, were covalently bound per g polymer. The modified polymer displayed improved internal cohesive properties due to the formation of interchain disulfide bonds within the polymer in aqueous solutions at pH-values above 5. Adhesion studies revealed strongly improved adhesive properties. Whereas the TWA was determined to be 104 ± 21 μJ for the unmodified polymer, it was 191 ± 47 μJ for the polymer-cysteine conjugate 16:1 and 280 ± 67 μJ for the polymer-cysteine conjugate 2:1.
Conclusions. Polymers with thiol groups might represent a new generation of mucoadhesive polymers displaying comparatively stronger adhesive properties.

KEY WORDS: mucoadhesion; cohesion; cysteine; polycarbophil; disulfide bonds.

INTRODUCTION

Since the concept of bioadhesion has been introduced into the pharmaceutical literature, many attempts in academia as well as industry have been undertaken to improve bioadhesive properties of various polymers. These attempts include the neutralization of ionogenic polymers (1), the precipitation of polymers in organic solvents and air drying instead of lyophillization (2), and the development of polymer-lectin conjugates (3,4), as well as, polymer-bacterial adhesin conjugates (5) focusing on a specific binding to epithelia. All these systems, however, are based on the formation of non-covalent bonds such as hydrogen bonds and ionic interactions. They are therefore able to provide only a weak adhesion being in many cases insufficient to guarantee the localization of a drug delivery system at a given target site. According to this, polymers capable of forming covalent bonds—even if it is only to the mucus layer—should display comparatively much higher adhesive properties.

The mucus layer covering GI-epithelia consists mainly of mucus glycoproteins which have a central region heavily laden with O-linked oligosaccharide chains and two flanking cysteine-rich subdomains on either side. These cysteine-rich subdomains containing over 10% Cys in their primary structure are involved in the linking of mucin monomers into oligomers via disulfide bonds, building up the three-dimensional network of the mucus gel layer (6). The mucolytic activity of thiols such as N-acetylcysteine is based on disulfide exchange reactions (7) between mucin glycoproteins in mucus and the mucolytic agent. Due to exchange reactions such as illustrated in Fig. 1, intra- as well as intermolecular disulfide bridges within the glycoprotein-structure are cleaved leading to a breakdown of the mucus. Based on the observation, that the mucolytic agent is thereby covalently bound to mucin glycoproteins in mucus, also other thiol bearing compounds in particular polymers with thiol groups should be covalently bound to the mucus (Fig. 1). Apart from this disulfide exchange reactions, the oxidative formation of additional disulfide bridges between thiol groups of the mucin glycoprotein and the polymer could be expected representing the principle of covalent chromatography for (poly)peptides on resins with thiol groups (9).

In order to verify this working hypothesis, it was the objective of this study to generate a polymer bearing thiol substructures and to demonstrate an improved mucoadhesion based on the formation of disulfide bonds between the modified polymer and the mucus gel layer. Cysteine was therefore covalently bound to polycarbophil (PCP) representing one of the most bioadhesive polymers (10). The mucoadhesive properties of the resulting polymer-cysteine conjugates should then be evaluated by different adhesion studies *in vitro*.

MATERIALS AND METHODS

Synthesis of Polymer-Cysteine Conjugates

The covalent attachment of cysteine to polycarbophil was achieved by the formation of amide bonds between the primary amino group of the amino acid and a carboxylic acid group of the polymer. Polycarbophil (Noveon AA1, BF Goodrich, Brecksville, Ohio, was neutralized with NaOH as described previously by our research group (11). Sixteen grams of neutralized polycarbophil (NaPCP) were hydrated in 4 L of demineralized water. The carboxylic acid moieties of the polymer were activated for 45 min by adding 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC; Sigma, St. Louis, Missouri, in a final concentration of 50 mM. In order to avoid oxidation of sulfhydryl groups by atmospheric oxygen, the pH-value was adjusted to 4–5 by adding 5 N HCl and the reaction mixture was gassed with nitrogen for 15 min. Increasing amounts of L-cysteine (Sigma, St. Louis, Missouri, as shown in Table I were added to 250 mL aliquots and reaction mixtures were incubated for 3 h at room temperature under nitrogen. According to the weight-ratio of polycarbophil to cysteine during this coupling reaction, the resulting polymer-cysteine conjugates were called 32:1 up to 1:4 as listed in Table I. The

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ABBREVIATIONS: EDAC, 1-ethyl-3-(3-dimethylamino propyl)carbodiimide hydrochloride; EDTA, ethylenediaminetetraacetic acid; MDF, maximum detachment force; TWA, total work of adhesion; NaPCP, polycarbophil neutralized with NaOH; TBS, Tris-HCl buffered saline (0.9% NaCl).

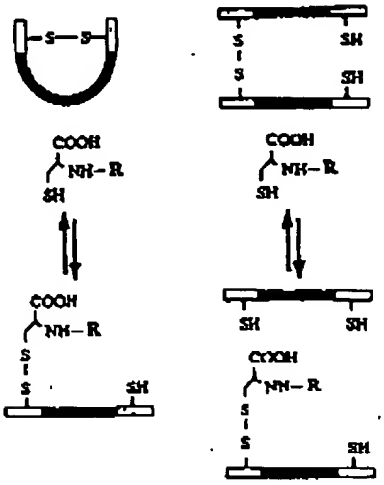

Fig. 1. Schematic presentation of disulfide exchange reactions between a (poly)peptide and a cysteine derivative according to G. H. Snyder (8). The (poly)peptide stands here for a mucin glycoprotein of the mucus and the cysteine derivative is a polymer-cysteine conjugate (R = polycarbophil).

conjugates were isolated by dialyzing at 10°C in the dark against 1 mN HCl containing 2 μM EDTA, two-times against the same medium but containing 1% NaCl and then exhaustively against 0.5 mN HCl. Samples being prepared and isolated in exactly the same way as polycarbophil-cysteine conjugates but omitting EDAC or cysteine during the coupling reaction served as control A and control B for the following analytical studies. The pH value of dialyzed polymer-cysteine conjugates and controls was adjusted to pH 5 with 2 N NaOH and samples were lyophilized by drying frozen aqueous polymer solutions at −30°C and 0.01 mbar (Christ Beta 1-8K; Osterode am Harz, Germany). Polymer-cysteine conjugates as well as controls were stored at 4°C until evaluation.

Determination of the Thiol Group Content

The degree of modification was determined by measuring the amount of thiol groups of polycarbophil-cysteine conjugates and corresponding controls using Ellman's reagent (DTNB, 5,5'-Dithiobis(2-nitrobenzoic acid), Sigma, St. Louis, Missouri). Nine milligrams of each conjugate were swelled for 2 h at room temperature in 1 mL of 100 mM phosphate buffer pH 8.2, 50 mN HCl and 4% NaCl. 100 μL of 0.5 N NaOH were added and aliquots of 200 μL transferred in the first wells of a microtitration plate (96-wells, not binding). After incubation for 45 min at room temperature with 100 μL of 0.4% (m/v) DTNB dissolved in 0.5 M phosphate buffer pH 7.1, absorbance at 405 nm was measured (Anthos Reader 2001, Salzburg, Austria). The amount of thiol groups was calculated using a standard curve obtained by the sulfhydryl group determination of a series of solutions containing unmodified polycarbophil and increasing amounts of cysteine.

Water-Absorbing Capacity

Thirty milligrams of lyophilized polycarbophil-cysteine conjugates and unmodified neutralized polycarbophil were compressed (Hanseaten Type EI, Hamburg, Germany) into 5.0 mm diameter flat-faced discs. The compaction pressure was kept constant during the preparation of all discs. Test discs were placed on a water permeable membrane serving as the bottom of a plastic tube with a diameter of 16 mm. The tube was then set in a vessel containing demineralized water of 20°C. At predetermined time points the amount of water uptake was calculated by re-weighing the tubing and content after removing the unbound water.

Disulfide Bond Formation Within the Polymer Conjugate

First, 20 mg of polycarbophil-cysteine conjugate 1:2 which had not been brought to pH 5 after dialyzing was hydrated in 1.6 mL of demineralized water for 12 h at 4°C. The pH-value of aliquots (0.8 mL) was then adjusted to pH 5.0 and pH 6.8, respectively, demineralized water was added in order to obtain a final volume of 1 mL and samples were incubated at 37°C under permanent shaking. At predetermined time points, aliquot

Table 1. Concentrations of Reagents Used for Reaction Mixtures in Order to Form Polycarbophil-Cysteine Conjugates with Increasing Amounts of Thiol Groups

Polycarbophil-cysteine conjugate	Polycarbophil (g/250 mL)	Added cysteine (g)	EDAC (mM)	Thiol groups (μMole per gram polymer); means ± S.D. n = 6–8
PCP-Cyst. 1:4	1	4	50	142.2 ± 38.0
PCP-Cyst. 1:2	1	2	50	12.4 ± 2.3
PCP-Cyst. 2:1	1	0.5	50	5.3 ± 2.4
PCP-Cyst. 4:1	1	0.25	50	3.7 ± 2.0
PCP-Cyst. 8:1	1	0.125	50	2.9 ± 1.4
PCP-Cyst. 16:1	1	0.0625	50	0.6 ± 0.7
PCP-Cyst. 32:1	1	0.03125	50	0.3 ± 0.5
Control A	1	0.03125 up to 4 g	—	0.0 ± 0.0
Control B	1	—	50	n.d.

Note: The degree of modification was determined using Ellman's reagent.

volumes of 150 µL were transferred to a microtitrationplate, the pH-value was adjusted to 8.2 with 1 N NaOH and 0.5 M phosphate buffer pH 8.2 was added in order to obtain a final volume of 200 µL. The amount of remaining thiol groups was then determined with Ellman's reagent as described above. In addition, the increase in viscosity due to the formation of interchain disulfide bonds was determined by measuring viscosity of the gel ($\Delta D = 10\ s^{-1}$/min; RotoVisco RT20, Haake GmbH, Karlsruhe, Germany) immediately after starting the reaction and after 8 h and 24 h of incubation at 37°C.

Mucin Binding Studies

First, 5 mg of porcine mucin (Sigma, St. Louis, Missouri) were dissolved in 1.0 mL of demineralized water. After the addition of 5 mg of the polycarbophil-cysteine conjugate 1:2 and unmodified neutralized polycarbophil, respectively, the pH-value was adjusted to 7.8 with 1 N NaOH and samples incubated for 2 h at 37°C while shaking. Samples were centrifuged for 10 min at 30.000 g and the supernatants containing unbound mucin discarded. The remaining pellets were diluted 1:10 with 50 mM Tris-HCl pH 7.8 containing 2% NaCl, again centrifuged and the supernatant removed. This purification step was repeated five times. Thereafter the amount of polymer bound mucin was spectrophotometrically (Lambda 16; Perkin-Elmer, Vienna, Austria) investigated by measuring the absorption shoulder at 280 nm.

In Vitro Evaluation of the Adhesive Properties

Tensile Studies with Dry Polymer Compacts

Thirty milligrams of lyophilized polycarbophil-cysteine conjugates, controls and unmodified neutralized polycarbophil were pressed to flat-faced discs as described above. The compaction pressure was kept constant during the preparation of all discs. Following this, tensiometer studies with these test discs were carried out on native porcine intestinal mucosa. Test discs were therefore attached to the mucosa with a force of 2.5 mN. After a contact time between test disc and mucosa of 30 min in 50 mM Tris-HCl buffered saline (TBS) pH 6.8 with and without 1% (m/v) dithiothreitol or 100 mM glycine-HCl pH 3.0 containing 0.9% NaCl at 25°C, the mucosa was pulled at a rate of 0.1 mm s^{-1} from the disc. The total work of adhesion (TWA) representing the area under the force/distance curve and the maximum detachment force (MDF) were determined using the WINWEDGE software in combination with EXCEL 5.0 (Microsoft).

Tensile Studies with Hydrated Polymers

In order to minimize the influence of an 'adhesion by hydration,' tensile studies were also carried out with hydrated polymers in a slightly modified way as described previously by Robinson and co-workers (12). 150 µL of aqueous gels of 2.5% (m/v) lyophilized NaPCP and polycarbophil-cysteine conjugate 8:1 were spread in a uniform monolayer over excised porcine intestinal mucosa which had been fixed on a flat surface (10 mm i.d.) exhibiting a relative weight of 0.26 g in system. In 100 mM TBS pH 6.8 at 25°C, the polymer was brought in contact with a second porcine mucosa. The TWA was then determined as described above.

Statistical Data Analysis

Statistical data analysis was performed using the *t* test with $p < 0.05$ as the minimal level of significance.

RESULTS

Synthesis of Polycarbophil-Cysteine Conjugates

For synthesis of polycarbophil-cysteine conjugates it was essential to avoid air oxidation of thiol groups. The coupling reaction was therefore carried out under nitrogen at a pH-value of 4–5. In order to remove Cu^{2+} -ions, which would catalyze an oxidation, EDTA was added in the first step of dialysis. Results demonstrated a good correlation between the polymer to cysteine ratio at the coupling reaction and the amount of covalently attached cysteine. The more cysteine was added to the polymer, the more covalently attached thiol groups could be determined in the resulting conjugate. The efficacy of the purification method described here has been verified by controls A. Omitting EDAC during the coupling reaction led to polymers exhibiting a negligible amount of cysteine. Results of this study are shown in Table 1. All polymer-cysteine conjugates were easy swellable in aqueous solutions at a pH-value above 5, thereby forming transparent gels of highly viscoelasticity. They are stable towards air oxidation as dry powders as well as in aqueous solutions at a pH-value below 5.

Swelling Behavior of Polymer-Cysteine Conjugates

Based on the theory of 'adhesion by dehydration' (13), the water uptake of the polymer-cysteine conjugate might also influence mucoadhesion. Water uptake studies, however, demonstrated no significantly quicker swelling behavior of the polymer-cysteine conjugates 32:1 up to 2:1. Merely the polycarbophil-cysteine conjugates 1:2 and 1:4 displayed a significantly higher water uptake in comparison to the unmodified polymer. Results of this investigation are shown in Fig. 2.

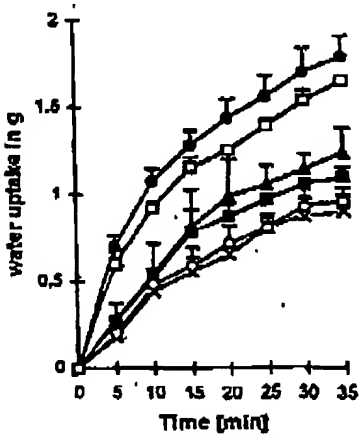

Fig. 2. Comparison of the water uptake of compacts (30 mg) of polycarbophil-cysteine conjugate 1:4 (●), polycarbophil-cysteine conjugate 1:2 (□), polycarbophil-cysteine conjugate 2:1 (▲), polycarbophil-cysteine conjugate 8:1 (■), polycarbophil-cysteine conjugate 32:1 (×), and unmodified neutralized polycarbophil (○). Represented values are means (±S.D.) of at least three experiments.

Formation of Disulfide Bonds Within Polymer-Cysteine Conjugates

In aqueous solutions at pH-values above 5 the thiol groups of polycarbophil-cysteine conjugates are not stable any more. They are continuously oxidized thereby forming disulfide bonds. The decrease in sulfhydryl groups at pH 6.8 is illustrated in Fig. 3. Due to the high density of carbonic acid moieties within poly(acrylic acid) derivatives, these polymers can also function as ion exchange resins. Hydrated matrix tablets based on such polymers are able to maintain a previously adjusted pH-value even in GI-fluids over several hours (data not shown). According to this, the formation of disulfide bonds within polymer-cysteine conjugates might be controllable by a priori adjusting the pH-value of the system. Whereas the amount of thiol groups decreases, viscosity of the polymer conjugate increases. Corresponding investigations demonstrated a viscosity of 2907 ± 193, 3228 ± 154 and 3394 ± 149 mPa*s (means ± S.D.; n = 4–5) after 0, 8 and 24 h of incubation at 37°C. This markedly increase in viscosity can be explained by the formation of interchain disulfide bonds leading to an improved cohesion of the polymer network. Adhesion of many quick swelling polymers is limited by an insufficient cohesion of the polymer resulting in a break within the polymer network rather than between the polymer and mucus layer. Although polycarbophil-cysteine conjugates are rapidly hydrated, they are able to form highly cohesive and viscoelastic gels due to the formation of additional disulfide bonds. Compacts of polycarbophil-cysteine conjugates, which were actually pressed for tensile studies, displayed high mechanical stability as well as elasticity without any erosion even after several days of incubation with 50 mM TBS pH 6.8. In contrast, compacts of unmodified polycarbophil disintegrated within several hours. Especially for polymer conjugates of high cysteine donation the formation of an over-hydrated slippery mucilage can therefore be completely excluded.

Mucin Binding Studies

The mucin is composed largely of flexible glycoprotein chains, which are crosslinked by disulfide bonds. Due to these disulfide bonds and/or remaining thiol moieties of the glycoprotein, it should be bound to polymers exhibiting sulfhydryl groups. Although a detailed quantification of the amount of mucin bound to tested polymers was impossible because of the heterogeneity of the used mucin, this theory could nevertheless be verified. Results demonstrated the mucin was effectively bound to the tested polymer-cysteine conjugate, whereas it was not at all bound to unmodified neutralized polycarbophil. Moreover, due to the addition of 1% (m/v) dithiothreitol already bound, mucin could be completely removed from the polycarbophil-cysteine conjugate.

Tensile Studies

Tensile studies with dry compacts of polymer-cysteine conjugate 32:1, 16:1, and 8:1 demonstrated a clear correlation between the amount of polymer-linked cysteine and the adhesive properties. The more cysteine was bound to the polymer, the higher were its adhesive properties. At the polymer-cysteine conjugates 8:1, 4:1, and 2:1 mucoadhesion reached a plateau phase displaying a more than twice as high total work of adhesion (TWA) than the unmodified polymer. A further increase in the amount of covalently linked sulfhydryl groups, however, lead to a comparatively lower TWA. A reason for this observation can be seen in a too strong modification of the original polymer leading also to a significantly higher swelling behavior as shown in Fig. 2. Results of adhesion studies are shown in Fig. 4. Whereas the maximum detachment force (MDF) of all conjugates and controls was in very good correlation with the total work of adhesion, it was comparatively higher at the polymer-cysteine conjugate 1:4. Tensile studies carried out at pH 3.0 instead of pH 6.8 revealed a significant decrease in the TWA of the polymer-cysteine conjugate displaying only a negligible amount of active thiolate anions at this pH-value.

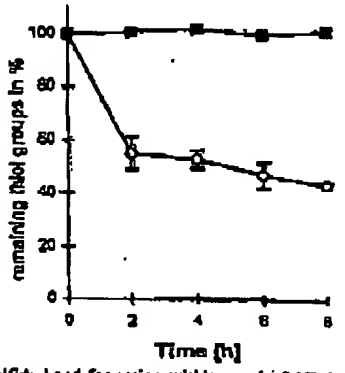

Fig. 3. Disulfide bond formation within a gel of 1% (m/v) polycarbophil-cysteine conjugate 1:2 at pH 6.8 (○) and pH 5.0 (●) at 37°C. Indicated values are means (±S.D.) of at least four experiments.

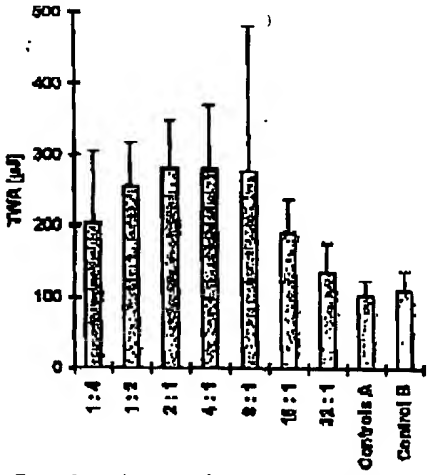

Fig. 4. Comparison of the adhesive properties of polycarbophil-cysteine conjugates and controls which were generated according to the scheme as listed in Table 1. Represented values are means ±S.D. (n = 5–8) of the TWA determined in tensile studies at pH 6.8 with dry compacts of indicated test material.

Whereas the increase in TWA of the polycarbophil-cysteine conjugate 2:1 was determined to be 2.69 ± 0.65-fold compared to unmodified polycarbophil at pH 6.8 (mean ± S.D.; n = 3), it was only 1.36 ± 0.71-fold at pH 3.0 (mean ± S.D.; n = 5). Furthermore, the increase in TWA of the same polycarbophil-cysteine conjugate compared to the unmodified polymer was also only 1.55 ± 0.23-fold at pH 6.8 (mean ± S.D.; n = 4) due to the addition of 1% dithiothreitol inhibiting the formation of disulfide bonds between the polymer and the mucus. The difference in TWA between the unmodified polymer and the polycarbophil-cysteine conjugate 2:1 was therefore neither at pH 3.0 nor in the presence of dithiothreitol of significance.

Tensile studies carried out with hydrated polymers demonstrated also an approximately twice as high TWA for the tested polycarbophil-cysteine conjugate. Results are shown in Table 2. As at this type of adhesion test, the break occurred more within the polymer itself than between the polymer and the mucus layer, it was impossible to differentiate between polymer adhesion and cohesion. Both factors, however, are essential for a long-term attachment of dosage forms to the mucosa.

DISCUSSION

According to our working hypothesis, the mucoadhesive properties of polymers should be improved due to the introduction of thiol groups leading to covalent bonds between the polymer and the mucus layer.

On the one hand this theory could be confirmed (I) by the effective immobilization of isolated mucin to the polymer-cysteine conjugate, whereas it was not at all bound to the unmodified polymer. (II) Tensile studies carried out with dry compacts of polymers demonstrated that the mucoadhesive properties of polycarbophil can be raised for more than 100% due to the immobilization of cysteine. (III) In contrast to tensile studies carried out at pH 6.8, the adhesive properties of the tested polycarbophil-cysteine conjugate 2:1 were strongly reduced at pH 3.0. At this pH-value the formation of disulfide bonds as well as disulfide exchange reactions can be almost excluded due to a negligible amount of negative thiolate anions, $-S^-$, representing the reactive form of cysteine in oxidation and nucleophilic attack (8). (IV) The comparably lower adhesive properties due to the addition of dithiothreitol suppressing the formation of disulfide bonds could also substantiate our working hypothesis.

However, we had to realize the improved adhesive properties of polycarbophil-cysteine conjugates cannot exclusively be explained by the formation of disulfide bonds between the polymer and the mucus layer. As the mechanism of mucoadhesion is even for well established mucoadhesive polymers not yet fully understood, the exact explanation of an additional mechanism turns out to be much more complex and difficult. In contrast to unmodified polycarbophil, for instance, the also quickly hydrated polycarbophil-cysteine conjugates remain very cohesive due to the formation of interchain disulfide bonds within the swelling polymer. The approximately twice as high TWA of the hydrated polycarbophil-cysteine conjugate 8:1 compared to the hydrated unmodified polymer has to be seen as the result of higher cohesive properties of the polymer conjugate, as the adhesive bond of both polymers failed more within the polymer itself. These results are in good accordance with earlier investigations demonstrating that the detachment of hydrated poly(acrylic acid) discs from a mucosa depends on interfacial phenomena as well as viscoelastic properties (14).

So far the use of quick swelling polymers was limited by an over-hydration leading to a slippery mucilage. Using such polymers the break occurred rather within the polymer than between the polymer and the mucus layer. In contrast, polycarbophil-cysteine conjugates display both high cohesive properties, which could be demonstrated in tensile studies carried out with hydrated polymers, and a quick swelling behavior. The results of this study revealed also a significantly improved swelling behavior of polycarbophil-cysteine conjugates 1:2 and 1:4 compared to the unmodified polymer. According to the theory, rapidly swelling polymers will also quickly interact with the mucin thereby providing good adhesion, the quicker water uptake of these polycarbophil-cysteine conjugates should also be taken into consideration as an additional effect for improved adhesive properties. However, in comparison to the polymer-cysteine conjugate 8:1 up to 2:1, which did not display a significantly improved swelling behavior, the adhesive properties of these two conjugates were even lower.

In summary, the high adhesive properties of polycarbophil-cysteine conjugates have therefore to be seen as a result of various factors. The influence of factors such as the formation of disulfide bonds, hydration and internal cohesion on the mucoadhesive properties of modified polymers can only be evaluated in connection with each other and not apart.

Table 2. Adhesive Properties of 2.5% (m/v) NaPCP and the Polymer-Cysteine Conjugate 8:1 at pH 6.8 According to the Method Described by Robinson and Co-workers (12)

Tested Polymer	Total work of adhesion (TWA) in μJ	± Standard deviation (n = 4–8)
PCP-Cysteine Conj. 8:1	8.81	2.94
NaPCP	4.05	1.27

CONCLUSIONS

The covalent attachment of cysteine to polycarbophil leads to polymer conjugates displaying strongly improved adhesive as well as cohesive properties. Being aware of the mucus turnover and peristaltism, these features should nevertheless render polycarbophil-cysteine conjugates useful as excipients for drug delivery systems such as tablets, pellets and microparticles providing a more prolonged residence time on various mucosal tissues compared to well established polymers.

ACKNOWLEDGMENTS

This work was supported by Grant No. P13085-MOB from the Fonds zur Förderung der wissenschaftlichen Forschung (FWF) to A. Bernkop-Schnürch. The authors wish to thank Mr. Strobel and co-workers from the slaughterhouse Totzenbach for supply of porcine intestinal mucosa.

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MS# 98/338 report number 98/338/c

Polymers with thiol groups: a new generation of mucoadhesive polymers?

by Bernkop-Schnürch, Schwarz V, Steiniger S

General comments

This is a well written article* making use of the cleavage of disulfide bonds by compounds containing thiols as acetylcysteine. This well known reaction is exploited to increase unspecific mucoadhesion by cleaving the disulfide bridge of mucus with acetylcysteine coupled to polycarbophil with the result that polycarbophil is covalently linked to mucus. This idea is brillant and there is some evidence given in the paper that it really works although the experimental in vitro circumstances especially if (synthetic) mucus is involved are very complex for a sound interpretation. The referee therefore suggests to include in this article simple ex-vivo methods as e.g. measuring of residence times of polymer-cysteine conjugates beads compared to polycarbophil beads in freshly isolated gut of rats or pigs as e.g. described by Lehr et al. in STP Pharma 5 (1989) 857-862 to have more evidence of improved mucoadhesion under physiological conditions.
Such a proof would also allow to omit the questionmark at the end of the title because then enough evidence is given that polymers containing thiol groups may be a new generation of mucoadhesive polymers if there are no toxicological contraints to use them.

* (the English could be improved by the desk editor and there are some minor typing errors).